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CHLOROPHYLLASE IN TISSUE CULTURES OF *KALANCHOË CRENATA*

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Abstract—Chlorophyllase (chlorophyll chlorophyllido-hydrolase) was not detected in dark-grown callus of *Kalanchoë* but was found in callus which had been exposed to light. The enzyme was present in detectable amounts before any chlorophyll was found. Chlorophyllase production was associated with the formation of lamellae. Chlorophyll was formed at a later stage in the development of the plastids. It was also observed that the longer time colourless callus was cultured in the dark the less was its ability to synthesize chlorophylls on transfer to the light.

INTRODUCTION

CHLOROPHYLLASE (chlorophyll chlorophyllido-hydrolase), which was first described in detail by Willstatter and Stoll,[1] catalyses the hydrolytic cleavage of chlorophyll *a* and chlorophyll *b* producing the corresponding chlorophyllide and free phytol.

Chlorophyllase has been isolated from a variety of plant materials[2,3] and is not specific for chlorophyll *a* and chlorophyll *b* but acts on a variety of related phytol esters. Fischer *et al.*[4] found that chlorophyllase hydrolysed bacteriochlorophyll and Sudyina[5] found that both phaeophytin *a* and phaeophytin *b* are cleaved by the enzyme to give free phytol and the corresponding phaeophorbide. The enzyme appears only to be active with those porphyrins which have a carboxymethyl group at carbon 10 and hydrogens at positions 7 and 8. *In vitro* it has been shown that the reaction is reversible and, by adjusting the concentrations of phytol and chlorophyllide *a*, chlorophyll can be synthesized.[6] On this evidence it has been suggested that chlorophyllase is responsible for the final reaction in the synthesis of chlorophyll *a in vivo*, that is, the esterification with phytol of the propionic residue at position 7 of chlorophyllide *a*. Further evidence which may implicate chlorophyllase in a synthetic rather than a degradative role was obtained by Holden[7] who found that etiolated pea leaves had a low chlorophyllase content compared with the fully green leaves. When the etiolated leaves were exposed to light, chlorophyllase activity increased, being paralleled by an increase in chlorophyll content. A comparable result was reported by Sudyina[5] who demonstrated a rapid initial increase in chlorophyllase activity in etiolated leaves exposed to light.

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1 R. WILLSTATTER and A. STOLL, *Investigations on Chlorophyll* (1913) (English translation by F. M. SCHERTZ and A. R. MERTZ), Science Press, Lancaster, Pa. (1928).
2 L. BOGORAD, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 29, Academic Press, New York (1965).
3 M. HOLDEN, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 461, Academic Press, New York (1965).
4 H. FISCHER, R. LAMBRECHT and H. MITTENZWEI, *Hoppe-Seylers Z.* **253**, 1 (1938).
5 E. G. SUDYINA, *Phtotochem. Photobiol.* **2**, 181 (1963).
6 S. SHIMIZU and E. TAMAKI, *Arch. Biochem. Biophys.* **102**, 152 (1963).
7 M. HOLDEN, *Biochem. J.* **78**, 359 (1961).

Events occur very rapidly when etiolated leaves are exposed to the light. A rapid synthesis of chlorophylls occurs.[8] Etioplasts complete their development to mature chloroplasts in etiolated leaves of *Phaseolus vulgaris* in about 45 hr.[9] The sequence of events in such a dynamic system may be obscured by the rapidity of the changes which occur. In callus cultures of *Kalanchoë crenata*, however, the normal conditions of growth are such that when colourless dark-grown callus is transferred to the light, chlorophylls are only formed slowly and increase during several months of subculture.[10] Thus, by using dark-grown callus of *Kalanchoë* and callus in various stages of greening, it seemed possible that correlations between changes in chlorophyllase activity and chlorophyll content could be determined more easily.

RESULTS

Acetone powders prepared from dark-grown callus, green callus, *Kalanchoë* leaves and *Vicia faba* leaves were assayed for chlorophyllase activity (Table 1). The chlorophyllase activity was less in green callus preparations than in preparations from the leaves when

TABLE 1. CHLOROPHYLLASE ACTIVITY IN ACETONE POWDERS PREPARED FROM CALLUS AND LEAVES

Material assayed	μg phaeophytin *a* hydrolysed in 4½ hr* per 100 mg acetone powder	μg phaeophytin *a* hydrolysed in 4½ hr* per mg chlorophyll
Kalanchoë		
Dark-grown callus	0·06	—
Green callus	6·2	22·0
Leaves	17·6	29·2
Vicia leaves	28·5	56·5

* Each result is the mean of five assays on each of five samples.

expressed per 100 mg acetone powder. The amount of chlorophyll in green callus was also low in comparison to that in *Kalanchoë* leaves.[10] However, if chlorophyllase activity was expressed on a per mg chlorophyll basis, the green callus tissue had similar levels to that in *Kalanchoë* leaves.

Negligible hydrolysis of phaeophytin *a* occurred in reaction mixtures containing acetone powders of dark-grown callus. The standard incubation period in these assays was for 4½ hr only but even after 24 hr chlorophyllase activity was still barely detected in the dark-grown callus preparations. Since chlorophyllase occurs in tissue such as roots,[11] seed coats[12] and albino leaves,[13] it seemed possible that small amounts of the enzyme might be present in dark-grown callus, in amounts not detectable in the direct assay using acetone powders. Enzyme purification procedures were therefore employed to investigate this point.

[8] E. C. SISLER and W. H. KLEIN, *Physiol. Plantarum.* **16**, 315 (1963).
[9] D. VON WETTSTEIN, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. 1, p. 19, Academic Press, New York (1966).
[10] A. K. STOBART, I. MCLAREN and D. R. THOMAS, *Phytochem.* **6**, 1467 (1967).
[11] H. MEYER, *Planta* **11**, 294 (1930).
[12] E. G. SUDYINA and G. G. LOZOVAYA, *Ukr. Bot. Zh.* **18**, 5 (1961).
[13] E. G. SUDYINA, *Ukr. Bot. Zh.* **18**, 2 (1961).

The suitability of various purification procedures was assessed using green callus as a comparison. The methods used by Holden[7] and Boger[14] did not prove successful, as enzyme activity was lost. The methods of Klein and Vishniac[15] gave an increase in specific activity of over a hundred-fold for chlorophyllase obtained from green callus (Table 2). This method, with slight modification, was used with dark-grown callus, but the pellet obtained on centrifuging the filtrate at 20,000 *g* (see Materials and Methods) was minute in comparison with that from green callus. To overcome this, it was found necessary to prepare first an acetone powder from the whole cells of the dark-grown callus and to suspend the powder in 10% v/v) isobutanol: water before extracting with buffer. The results show however, that there was no increase in the specific activity of chlorophyllase from dark-grown callus. To test for possible inhibitors of chlorophyllase activity in extracts of dark-grown callus, chlorophyllase

TABLE 2. PURIFICATION OF CHLOROPHYLLASE

Material assayed	Protein (mg)	μg phaeophytin *a* hydrolysed per mg protein per hr
Green callus		
Sodium deoxycholate supernatant	18·0	1·7
NH_4SO_4 precipitate	0·6	198·0
Dark-grown callus		
Sodium deoxycholate supernatant	6·3	0
NH_4SO_4 precipitate	0·02	0

TABLE 3. TESTS FOR INHIBITORS OF CHLOROPHYLLASE ACTIVITY IN EXTRACTS OF DARK-GROWN CALLUS*

Green callus extract assayed	μg phaeophytin *a* hydrolysed* per mg protein per hr
Alone	196
With dark-grown callus extract	192

* Each result is the mean of five assays.

in green callus extracts was assayed in the presence of dark-grown callus extracts; no diminution of activity occurred (Table 3) so it seemed unlikely that inhibitors of chlorophyllase activity were present.

It was known from previous work in this laboratory that greening occurred when dark-grown callus was transferred to 16-hr days. The chlorophyll content increased slowly in the callus, subcultured at monthly intervals, over a period of several months in the light. If chlorophyllase played a role in the phytylation of chlorophyllide *a* to produce chlorophyll *a*, then enzymic activity might be expected to increase in parallel with increasing chlorophyll content. Dark-grown callus was subcultured onto fresh medium, transferred to the light, and was both subcultured and assayed for chlorophyllase activity at 4-weekly intervals for 20 weeks. It was apparent (Fig. 1) that chlorophyllase was produced during the first 4 weeks in the light, that is in first generation callus, and no chlorophyll was detected at this time. The

[14] P. BOGER, *Phytochem.* **4**, 435 (1965).
[15] A. KLEIN and W. VISHNIAC, *J. Biol. Chem.* **236**, 2544 (1961).

chlorophyllase activity did not substantially increase in second-generation callus (8 weeks in the light) and chlorophyll was still absent. However, in subsequent generations the chlorophyllase activity showed a marked increase and paralleled the increase in total chlorophyll. The activity of chlorophyllase expressed per mg chlorophyll was similar for all chlorophyll-containing callus assayed. These results suggest a correlation between the appearance of chlorophyllase and the synthesis of chlorophyll.

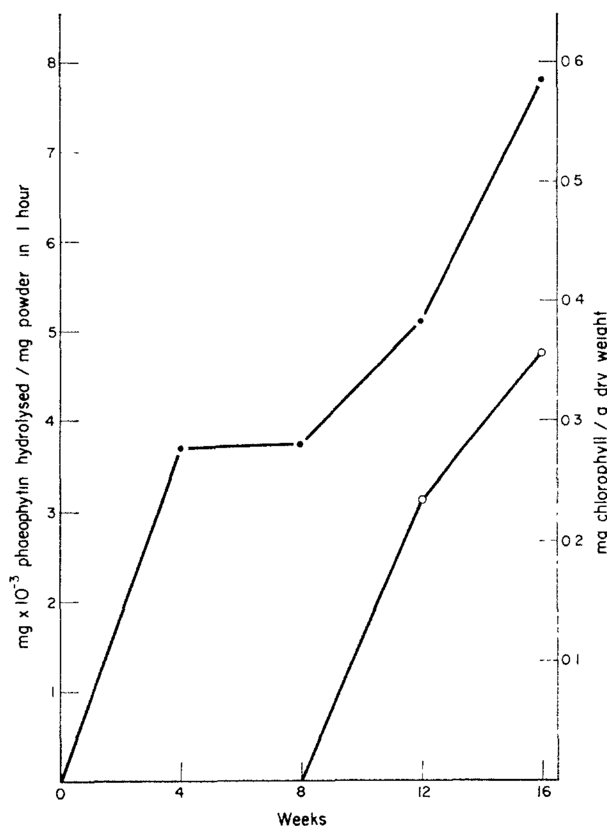

FIG. 1. CHLOROPHYLL PRODUCTION AND INCREASE IN CHLOROPHYLLASE IN DARK-GROWN CALLUS TRANSFERRED TO 16-hr DAYS

●—● chlorophyllase; ○—○ chlorophyll. Chlorophyllase activity, expressed as μg phaeophytin hydrolysed per mg chlorophyll per hr, after 12 weeks = 22·2; after 16 weeks = 22·1.

Dark-grown callus was transferred to 16-hr days and grown in the light for 4 weeks, i.e. first-generation callus. This callus was fractionated and the chlorophyllase activity was assayed in acetone powders prepared from the 3000 *g* and 20,000 *g* fractions. Similarly, green callus was fractionated and the acetone powders of the 3000 *g* and 20,000 *g* fractions assayed for chlorophyllase. Chlorophyllase activity was detected in fractions of each type of callus (Tables 4 and 5). When enzyme activity was expressed on a chlorophyll basis, the chlorophyllase activity of the green callus fractions showed a two-fold increase over the activity for the enzyme in acetone preparations from whole cells. This difference may be attributed, in part, to adsorption of the enzyme onto cell wall and other debris present in the

whole-cell acetone powder preparations and also to adsorption of phaeophytin *a* or phaeophorbide *a*. It seems likely, however, that partial purification of the enzyme was achieved by fractionation of the green callus, leading to increased enzyme activity. The activity per unit chlorophyll in the 3000 *g* and 20,000 *g* fractions was similar. These results indicate that chlorophyllase was segregating with the chlorophyll-bearing membranes. No chlorophyll was detected in fractions of first-generation callus but chlorophyllase activity was associated with both the 3000 *g* and 20,000 *g* fraction, more activity being detected in the 20,000 *g* fraction. Possibly, for first-generation callus, the 20,000 *g* fraction consists of small plastids, the precursors of chloroplasts, together with plastid fragments and mitochondria. Larger plastids, probably containing rudimentary structures of developing chloroplasts, might have sedimented in the 3000 *g* fraction.

TABLE 4. CHLOROPHYLLASE ACTIVITY IN GREEN CALLUS FRACTIONS

Extract assayed	μg phaeophytin hydrolysed per mg* chlorophyll per hr
Whole green callus	21·7
3000 *g* fraction	40·9
20,000 *g* fraction	41·0

* Each figure is the mean of five assays on two samples.

TABLE 5. CHLOROPHYLLASE ACTIVITY IN FRACTIONS OF FIRST-GENERATION CALLUS

Extract assayed	μg phaeophytin hydrolysed per hr per 100 mg acetone powder*
Whole tissue	0·36
3000 *g* fraction	0·43
20,000 *g* fraction	0·61

* Each figure is the mean of five assays on two samples.

The dark-grown callus used throughout this work was the "clone" established by McLaren and Thomas[16] from *Kalanchoë* stems and which had been subcultured at monthly intervals and grown in the dark for 2 years.

An interesting feature was that this dark-grown callus on transfer to the light did not produce detectable amounts of chlorophyll until it had been 8, and sometimes 12 weeks, in the light. This was in contrast to the results reported previously,[10] when dark-grown callus produced detectable amounts of chlorophyll after only 4 weeks in the light. The dark-grown callus from *Kalanchoë* stems used in the earlier experiments of Stobart *et al.*[10] had spent only 6 months in the dark before transfer to the light. It seemed necessary therefore to reinvestigate the production not only of chlorophylls but also of carotenoid pigments in dark-grown callus on transfer to light. Pigment analyses on the 2-year-old dark-grown callus were therefore compared with those obtained in earlier work.

On transfer to 16-hr days with subculturing at monthly intervals, total chlorophyll rapidly increased in the 6-month-old callus as compared with a slower rate of chlorophyll

[16] I. McLaren and D. R. Thomas, *New Phytol.* **66**, 683 (1967).

formation in 2-year-old callus (Fig. 2). The time taken for the chlorophyll *a*/chlorophyll *b* ratio to approach that for *Kalanchoë* leaves also differed. Six-month-old callus approached the leaf ratio within 4 months but 2-year-old callus was 6 months in the light before it attained leaf ratio values. A similar picture emerged for the carotenoid analysis (Fig. 3). Xanthophyll

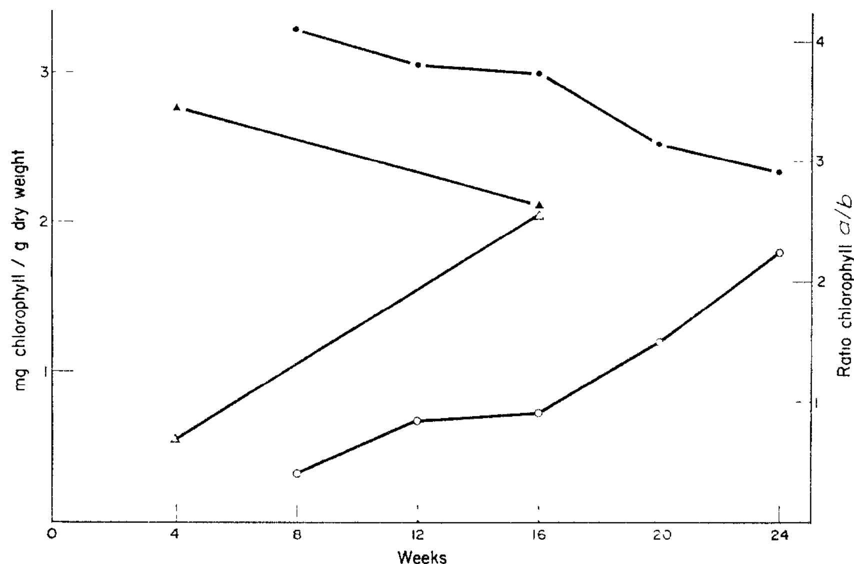

FIG. 2. CHLOROPHYLL PRODUCTION IN DARK-GROWN CALLUS ON TRANSFER TO LIGHT.
○—○, 2-yr callus; △—△, 6-month callus. Transfer to 16-hr days. Chlorophyll *a*/*b* ratios, ●—● for 2-year-old callus and ▲—▲ for 6-month-old

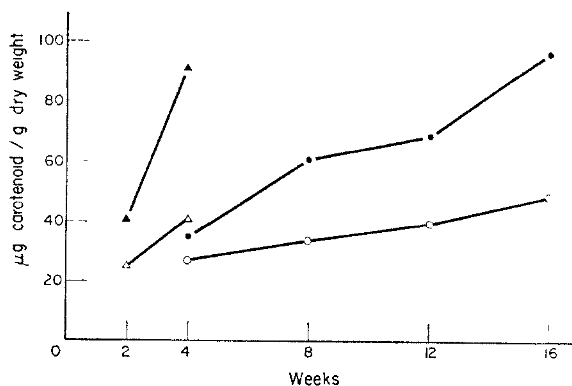

FIG. 3. CAROTENOID PRODUCTION IN DARK-GROWN CALLUS TRANSFERRED TO 16-hr DAYS.
β-Carotene production in ○—○, 2-year-old callus; △—△, in 6-month-old callus. Xanthophyll production in ●—●, 2-year-old callus; ▲—▲, in 6-month-old callus.

and β-carotene production in 6-month-old callus was faster than that in 2-year-old callus. Thus it would seem that the length of time previously spent in the dark by the callus diminished the ability of the callus to form the chloroplast pigments on transfer to the light. Nevertheless the 2-year-old dark-grown callus still retained the ability, albeit decreased, to become green in the light.

The electronphotomicrographs of plastids from first-generation callus showed that the plastids were of a simple structure. Many plastids contained prominent starch grains (Plate 1*a*) and vesicles were present in all plastids. Some vesicles situated near the perimeter were in continuity with the plastid inner membrane. Whilst many vesicles were present in the stroma, lamellae, stacked loosely together, gave the appearance of an early stage in grana development. Characteristically, the stroma of all first-generation callus plastids were stained heavily. This might have been due to the presence of many ribosomes, and treatment of plastid sections with RNAase[17] would perhaps have confirmed this.

In the green callus, chloroplasts of a more advanced structure were observed (Plate 1*b*). Thylakoids were stacked into grana which were bounded top and bottom by a single membrane. The lamellae of the grana appeared in some cases to be continuous with the plastid inner membrane although a really convincing continuity was not demonstrated. Frequent invaginations of the inner membrane formed vesicles, some flattened, in the stroma.

DISCUSSION

It is well known that morphological, cytological and metabolic changes may occur in plant tissue cultures during prolonged culture.[18–20] In the present case it is clear that the longer dark-grown callus of *Kalanchoë* underwent subculture and growth in the dark, the less was its ability to synthesize chloroplast pigments. It is possible that the reproduction of plastids, which in the light become chloroplasts, did not keep pace with division of cells in dark-grown callus and thus the chloroplast precursors became "diluted" by growth. However, cells of first generation callus contained many plastids of known structure (Plate 1*a*). Dark-grown callus cells contain plastids of simpler structure[21] but on exposure of the dark-grown callus to light the plastids did not rapidly synthesize pigments (Figs. 2 and 3), nor did they develop quickly into mature chloroplasts but remained simple in structure. If they had developed rapidly, then chlorophyll should have been detected in first-generation callus and the development of a more complex structure might have been expected, as a result of plastid maturation. That chlorophyll was not detected and that plastids were at a juvenile stage in development seems to favour the suggestion that the ability of the cells to produce vital genetic or hormonal regulators of plastid growth gradually decreased, was impaired or was selected out during sustained growth in the dark.

Chlorophyllase activity increased with the development of plastids in dark-grown callus exposed to the light. Chlorophyllase was detected before chlorophylls in the callus. A net synthesis of chlorophyll occurred during the time when chlorophyllase activity was increasing in the callus. Chlorophyllase activity expressed on a chlorophyll basis was similar for all callus which contained chlorophyll. These correlations can provide only indirect evidence for an *in vivo* role of chlorophyllase, but it may be inferred that the enzyme plays a part in chlorophyll synthesis and is responsible for the phytylation of chlorophyllide. However, Perkins and Roberts[22] demonstrated a turnover of chlorophyll in mature leaves of some dicotyledon species and if a hydrolytic role is assigned to chlorophyllase then the enzyme may

[17] N. Kislev, H. Swift and L. Bogorad, in *Biochemistry of Chloroplasts* (edited by T. W. Goodwin), Vol. 1, p. 355, Academic Press, New York (1966).
[18] G. P. Hill, *Ann. Bot.* **31**, 437 (1967).
[19] H. E. Street, in *Cells and Tissues in Culture* (edited by E. N. Wilmer), Vol. 3, p. 533, Academic Press, New York (1967).
[20] J. G. Torrey, *Physiol Plantarum* **20**, 265 (1967).
[21] A. T. Pickering and D. R. Thomas (in preparation).
[22] H. J. Perkins and D. W. A. Roberts, *Can. J. Botany* **41**, 221 (1963).

play a part in such a turnover of chlorophyll. Aradoo and Vennesland[23] associated chlorophyllase with the lipoprotein membranes of spinach chloroplasts. Fractionation of first-generation and green callus showed that the enzyme was present in a particulate fraction. It seemed therefore that chlorophyllase activity was probably located on the lipoprotein membranes in the plastids of first-generation callus. Possibly some enzyme in such juvenile plastids was distributed in lipoprotein aggregates in the stroma, as the dense staining of the stroma might indicate not only the presence of many ribosomes but also small aggregates of lipoprotein. Further fractionation studies on isolated juvenile plastids are required to clarify this point as it is not without significance in consideration of the origin of at least some of the lamellae in the developing plastids. That is, some lamellae may well result from a *de novo* condensation in the stroma as well as from invagination of the inner membrane.

Chlorophyll synthesis does not appear to be a requirement for the development of lamellae in the plastids, as first-generation callus plastids contain lamellae but no chlorophyll. A similar conclusion was reached by von Wettstein working with genetic mutants of barley,[24] but he concluded that chlorophyll formation was required for the "normal arrangement of lamellae," presumably into grana, in the chloroplast. Although a juvenile stage of grana development seemed evident in first-generation callus plastids, normal arrangement of lamellae into grana was not observed. In the green callus which possessed chlorophyll and contained photosynthetically active chloroplasts,[16] thylakoids stacked into grana were observed. Thus if a synthetic role is assigned to chlorophyllase then its early presence on the lipoprotein lamellae might be an important determinant in chloroplast development.

MATERIALS AND METHODS

General Techniques

Culture methods, pigment extraction procedures and chlorophyll determinations were as previously described.[10,16] Carotene and xanthophyll fractions were separated from saponified pigment extracts on a column of Hyflo-supercel. Carotenes were eluted with light petroleum (40–60°) and xanthophylls, which remained on the column, were eluted by peroxide-free diethyl ether. Carotenes were estimated using the $E_{1}^{1\%}$ cm value for β-carotene and xanthophylls were estimated using the $E_{1}^{1\%}$ cm value for lutein.[25]

Acetone Powders

Acetone powders of callus and leaf tissue were prepared by the method of Loomis.[26] Acetone and ether extracts obtained in the preparation of acetone powders were bulked and the acetone and ether removed by evaporation under N_2. Residual pigments were extracted into ether and the chlorophyll content determined.[27] Protein contents of enzyme preparations were measured by the Biuret method.[28]

Fractionation of Green Callus

All the ensuing operations were conducted at 2° in a cold room. Callus tissue was washed in liquid medium and carefully dried on a Buchner funnel under slight suction. The callus,

[23] A. C. Aradoo and B. Vennesland, *Plant Physiol.* **35**, 368 (1961).
[24] D. von Wettstein, in *Brookhaven Symposia in Biology* **11**, The photochemical apparatus, p. 138 (1958).
[25] B. D. Davies, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. Goodwin), p. 489, Academic Press, New York (1965).
[26] W. D. Loomis, *Plant Physiol.* **34**, 541 (1959).
[27] C. L. Comar and F. P. Zscheile, *Plant Physiol* **17**, 106 (1942).
[28] E. Layne, in *Methods in Enzymology* (edited by S. P. Colowick and N. O. Kaplan), Vol. III, p. 477, Academic Press, New York (1957).

with an equal volume of acid-washed sand, was transferred to a pre-cooled mortar and covered with 0·35 M NaCl buffer, pH 7·4, containing 0·01 M K_2HPO_4 and 0·01 M EDTA. After grinding for 5 min, the brei was filtered through eight layers of fine muslin. The filtrates were centrifuged at 200 *g* for 2 min to remove most cell debris. The supernatants were centrifuged at 3000 *g* for 7 min. The pellets were resuspended in fresh cold buffer solution and again centrifuged at 3000 *g* for 7 min. The supernatants from both 3000 *g* runs were bulked and centrifuged at 2000 *g* for 30 min. Acetone powders were prepared from the pellets obtained at 3000 *g* and 20,000 *g* and these powders were assayed for chlorophyllase.

Chlorophyllase Assay

Chlorophyllase was assayed in aqueous solution using phaeophytin *a* as substrate.

Chromatographically pure phaeophytin *a* for the assays was prepared from chlorophyll *a* isolated from spinach leaves. The assays were conducted in graduated centrifuge tubes. Weighed quantities of acetone powder were suspended in 2 ml 0·8 M phosphate buffer, pH 7·5, containing 0·2% (v/v) Triton × 100 and equilibrated for 10 min at 30°. 30 μg phaeophytin *a* dissolved in 0·05 ml peroxide-free diethyl ether was added to start the reaction which was continued for 4·5 hr. The reaction was stopped by the rapid addition of 4 ml acetone and 6 ml light petroleum (60–80°). The centrifuge tubes were capped, well shaken and the phases resolved by centrifugation. The volume of each phase was recorded and each phase removed separately with a finely drawn out glass pipette and transferred to 1 ml glass cuvettes. The optical density at 667 nm was measured on a Unicam SP500 spectrophotometer and the concentrations of phaeophytin *a* (light petroleum phase) and of the phaeophorbide *a* (acetone phase) calculated using the absorption coefficients given by Klein and Vishniac.[15]

Enzyme Purification

Purification of chlorophyllase in acetone powders of callus tissues was attempted using three distinct procedures.[7,14,15] The only method which proved successful was adopted from the work of Klein and Vishniac.[15] Acetone powders of callus tissues were suspended in 10% (v/v) isobutanol with gentle stirring for 30 min and supernatants discarded. The suspension was centrifuged at 15,000 *g* for 30 min and the supernatants discarded. The pellet was resuspended in aqueous 1% (w/v) sodium deoxycholate and left at 0° for 30 min. The solution was centrifuged at 20,000 *g* for 20 min and the supernatants retained. Extraction of the pellets with 1% (w/v) sodium deoxycholate was repeated twice. The supernatants were bulked and made 30% saturated with respect to ammonium sulphate. The protein precipitate was collected by centrifugation, washed with cold 30% saturated ammonium sulphate solution and finally dissolved in a small volume of 0·1 M phosphate buffer, pH 7·5. In assays for chlorophyllase activity 0·1 ml enzyme solution was made up to 2 ml with the reaction mixture.

Electron microscopy[29]

Small callus lumps were fixed for 2 hr at 4° in 0·1 M phosphate buffer, pH 7·3, containing 5% (v/v) gluturaldehyde and transferred to veronal–acetate buffer, pH 7·3 (2·94 g sodium veronal, 1·94 g CH_3COONa, $3H_2O$ in 100 ml water) and washed overnight. The callus lumps were then immersed in 1% (w/v) OsO_4 in veronal–acetate buffer (pH 7·3) for 2 hr. The callus was dehydrated in the ethanol–propylene oxide series and embedded in araldite resin.

[29] D. H. Kay, *Techniques for Electron Microscopy*, Blackwell, Oxford (1965).

Sections were cut on an L.K.B. ultramicrotome and when on the grid were stained with uranyl acetate and lead citrate.

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